Chapter 9

Protein synthesis and antioxidant capacity in ageing mice: effects of life-long cold exposure

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Abstract

Substantial evidence supports a key role for reactive oxygen species (ROS) in causing cumulative damage to cellular macromolecules, thereby contributing to senescence. Antioxidants can scavenge ROS while protein turnover removes and replaces oxidized proteins. How these defence systems vary with age and with metabolic demand is not well known. In the present study 2H5-phenylalanine was injected into young (3 months) and old (27 months) mice chronically exposed to cold to explore effects of cold exposure on age-related changes in liver and muscle protein synthesis. In addition, effects of cold exposure (10∞C) on antioxidant enzyme activities were investigated in two metabolically active tissues in mice at various ages (3-27 months). Cold exposure did not affect fractional synthesis rates (FSR) in liver or muscle. FSR rates did decrease with age in both tissues, and in liver this occurred more rapidly in cold-exposed animals than in controls. Antioxidant enzyme activity (SOD and GPx) was also affected by age. SOD activity peaked in 11 month old mice followed by a decline, while GPx activity slowly declined with age. SOD activity in heart was unaffected by cold. In liver, SOD activity was decreased in cold-exposed animals, but GPx activity was not. No relationship between energy expenditure and enzyme activity was found. Cold exposure increased metabolic rate by approximately 40% with no concurrent increase in antioxidant enzyme activity. Cold-exposed animals did not up-regulate either protein synthesis or antioxidant enzyme activities to provide protection against extra production of ROS.

INTRODUCTION

Reactive oxygen species (ROS) are produced as by-products of aerobic metabolism in mitochondria. They can cause damage to macromolecules (lipids, DNA and proteins) (Beckman and Ames, 1998; Davies *et al.*, 1982; Mecocci *et al.*, 1999; Tyler, 1975), and thereby contribute to senescence and several degenerative diseases associated with ageing (e.g. cardiovascular disorders, Parkinson disease) (McEwen *et al.*, 2005; Melov *et al.*, 1999; Wallace, 2005). An elaborate defence system consisting of endogenous antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and numerous non-enzymatic antioxidant (vitamins, flavenoids), exist that scavenge ROS to prevent deleterious effects (Beckman and Ames, 1998). Antioxidant enzymes cannot scavenge all the ROS produced; a small part escapes conversion and can damage proteins. This will impact on essential functions within the cell, such as maintenance of the structural architecture and enzyme activity. Indeed, the accumulation of oxidized proteins with age has been reported for many experimental ageing models (Stadtman, 2004). Such accumulation could be due either to increased generation of reactive oxygen species or to reduced elimination of oxidized proteins (Shringarpure and Davies, 2002; Stadtman, 2004).

Protein turnover, the composite of protein degradation (removal of inactive or damaged proteins) and protein synthesis (replacement with new, undamaged proteins) plays a potentially vital role in the ageing process (Rattan, 1996; Ryazanov and Nefsky, 2002; Sohal, 2002). Maintaining high rates of protein synthesis in old age could diminish the accumulation of damaged proteins and increase cell survival. Indeed, protein turnover is increased in calorically-restricted animals (Lambert and Merry, 2000; Lewis *et al.*, 1985), a nutritional condition that increases life span in several species, including mice (Weindruch *et al.*, 1986), and rats (McCay *et al.*, 1935), for recent reviews see (Masoro, 2005; Merry, 2005). Other factors can also influence protein synthesis and protein degradation, including cold exposure (rats: (McAllister *et al.*, 2000; Samuels *et al.*, 1996); calves: (Scott *et al.*, 1993), chickens: (Yunianto *et al.*, 1997)) and age (mice: (Blazejowski and Webster, 1983); rats: (Lewis *et al.*, 1985) and humans: (Short *et al.*, 2004; Young *et al.*, 1975); for reviews see: (Dorrens and Rennie, 2003; Rattan, 1996; Van Remmen *et al.*, 1995; Ward, 2000)).

Protein turnover accounts for 20-35% of the resting metabolic rate at thermoneutrality (Lobley, 1990; Newsholme, 1987). It has been hypothesised that protein turnover would increase in cold-exposed animals to cope with the increased demands on heat production (McAllister *et al.*, 2000). Indeed, in hearts of rats exposed to cold for 21d an increase in protein synthesis rates was shown (McAllister *et al.*, 2000), although contradictory results have also been reported (Lindsay *et al.*, 1988; Scott *et al.*, 1993; Yunianto *et al.*, 1997). Cold-exposure increases metabolic rate and ROS production and this may counteract any beneficial effect of increased protein breakdown. Alternatively, animals may adapt to the extra

ROS production by increased activity of antioxidant enzymes and, in concert with elevated protein breakdown, protect cells from problems arising from the presence of oxidized proteins. Such protection may vary with age, however, due to altered responsiveness in protein turnover and antioxidant systems.

The current study addresses some of these questions. First, the impact of cold-exposure on age-related changes in protein synthesis and the antioxidant enzyme system was monitored in C57BL mice exposed to 10°C and 22°C throughout life. This included comparisons between liver and muscle (protein synthesis) and liver and heart (anti-oxidant activity). Second, the relationship between metabolism and antioxidant enzyme activity between individual animals was examined.

MATERIAL AND METHODS

Animals & housing

Male C57bl6J mice were obtained from Harlan Nederland B.V. at the age of four weeks and individually housed in standard cages (15x30x15 cm, Macrolon type II, UNO Roestvaststaal BV, Zevernaar, NL) with standard bedding (Hemparade®, HempFlax, Oude Pekela, NL; and Envirodry®, BMI, Helmond, NL). Food (Standard rodent chow RMB-H (2181), HopeFarms, Woerden, NL) and water were provided *ad libitum*. Animals were divided over two rooms and housed at an ambient temperature of 22°C (Control group; WARM) or 10°C (Cold group; COLD). Animals were tested and sacrificed at four ages: 3, 11, 19, and 27 months.

Tissue collection

At each sacrifice age, five to eight mice per group were lightly anaesthesized with CO_2 and then killed by decapitation. Samples of liver and heart were quickly removed and immediately frozen in liquid nitrogen and stored at –80°C for antioxidant measurements.

Protein synthesis was assessed only in 3 and 27 month old animals. The latter were the same animals used for the antioxidant measurements, but for the 3 month old group different animals were used (n=8 per group). Food intake (g d^{-1}) and body mass was measured for two consecutive days prior to the sampling of tissues for protein synthesis measurements. Protein synthesis was measured using the large-dose method as described by Garlick *et al.* (1980). Mice were given an intraperitoneal injection of 150 mM $^2H_5$-phenylalanine (1,5 ml per 100 g animal). After 15 minutes the mice were euthanized using CO_2, followed by decapitation. Trunk blood was collected in pre-chilled tubes with heparin as anti-coagulant. Blood samples were centrifuged at 2600 g at 4°C for 15 min, and the plasma was collected and stored at –80°C until analysis. Liver and hind-leg muscle were rapidly removed, weighed to 4 decimal places, rinsed in ice-cold saline, frozen in liquid nitrogen, and stored at –80°C until analysis. Exact times (nearest second) of injection and freezing of tissues were recorded.

Protein synthesis

Free and protein-bound enrichments of phenylalanine in liver and muscle tissues were quantified as described by Wester *et al.* (2004) (Wester *et al.*, 2004). Approximately 300 mg of frozen tissue was homogenised on ice in 3 ml 7% (w/v) sulphosalicylic acid (SSA). Free phenylalanine was separated from protein-bound phenylalanine by centrifugation at 1000 g at 4°C for 15 minutes and the supernatant retained. The pellet was then washed three times with 3 ml 7% sulphosalicylic acid to remove free phenylalanine. The initial supernatant fraction (free pool) was passed through a 0.4 ml column of Dowex AG 50W-X8 (100-200 mesh) and the resin rinsed with 2x3.5 ml water before the phenylalanine was eluted with 2 ml 2M NH4OH and 1 ml water. The eluate was freeze-dried and stored at –20°C for later analysis. Half of the washed pellet (protein-bound pool) was transferred to a 8 ml screw-topped Pyrex hydrolysis tube and solubilised in 1 ml 0,5 ml 0,3 M NaOH for 30 minutes. A few phenol crystals were added and the sample was hydrolysed by adding 7 ml 4M HCl and heating on a dri-block at 110°C for 18 hours. Hydrolysates were dried under vacuum, resuspended in 1 ml 0,5 M sodium citrate (pH 6.2), and stored at -80°C until later analysis.

For the plasma samples, 150 ml was treated with 150 ml 15% SSA, centrifuged at 1000 g at 4°C for 10 minutes and 150 ml of the supernatant was passed through a 0.2 ml column of Dowex AG 50W-X8. Elution conditions and subsequent treatments were similar to the tissue free pool samples.

STable isotope enrichments of the tissue and plasma free pools were measured by gas chromatography mass spectrometry (GC/MS) after conversion to the tertiary-butyldimethylsilyl (TBDMS) derivatives (Calder and Smith, 1988). In the hydrolysed samples (protein-bound pool, low enrichment) phenylalanine was converted to phenylethylamine by enzymatic decarboxylation prior to forming the TBDMS derivative. This was separated by capillary column gas chromatography and enrichments obtained from electron impact ionization selective ion monitoring (EI-SIM) mass spectrometry (see Calder *et al.*, 1992 and Slater *et al.*, 1995).

The fractional synthesis rate (FSR, % d^{-1}) was calculated using the following equation: FSR = 100*(BP/FP)*1440/t, where BP is the bound pool of phenylalanine in mole percent excess (MPE), FP is the MPE of the free pool of phenylalanine measured in either plasma or tissue, and t is the time (min) between injection of phenylalanine and freezing of the tissue in minutes. The ratio between FP measured in plasma and liver or muscle was calculated to determine whether the injected phenylalanine had equally mixed with both free pools. The ratio was 1.04±0.07 and 0.99±0.08 (mean±sd) in muscle and liver respectively. Neither ratio was significantly different from unity. From these data it was concluded that the injected phenylalanine effectively equilibrated between tissue and plasma and remained so until time of death. In consequence, the FSR reported represent the FSR calculated based on plasma free phenylalanine as representative of the precursor pool.

Antioxidant enzyme activities & protein content

Prior to enzyme activity determinations, tissue samples were homogenized by sonication in 20 volumes of ice cold 50 mM phosphate buffer. Following centrifugation (25 min at 3000g), the supernatant fraction was collected, divided over several tubes and stored at –80°C for enzyme activity and protein measurements.

Total superoxide dismutase (SOD) activity was determined at 25°C by the inhibition of the auto-oxidation of pyrogallol by SOD in the supernatant, following the method of Marklund and Marklund (Marklund and Marklund, 1974). The reaction was followed spectrophotometrically at 420 nm in the following reaction mixture: 50 mM Tris-DTPA buffer, 15 μl supernatant and 15 μl pyrogallol in a total volume of 800 μl. Each triplicate measurement was preceded by a blank, containing only pyrogallol in Tris-DTPA buffer. One unit of SOD was defined as the amount of enzyme causing 50% inhibition of pyrogallol auto-oxidation.

Glutathione peroxidase (GPx) activity was determined at 25°C via the oxidation of NADPH in the presence of reduced glutathione (GSH) and H_2O_2 (combining the assays of Paglia and Valentine (1967), and Lawrence and Burk (1976)). The following reaction mixture was used: 4.28 mM sodium azide (to block catalase activity), 1.07 mM EDTA, 4.286 mM GSH, 0.214 mM NADPH, 1 U ml^{-1} GR in ice cold 50 mM phosphate buffer. 25 μl H_2O_2 and 25 μl sample were added to the reaction mixture. Reactions were followed spectrophotometrically at 340 nm in a total volume of 700 μl. To correct for spontaneous oxidation reactions independent of GPx, blanks without H_2O_2 were measured and subtracted from the assay values. One unit of GPx was defined as the amount of enzyme that oxidized 1 μmol of NADPH per minute in the presence of reduced glutathione. Protein content of the supernatant fraction was determined using a Bradford assay (Quick start Bradford protein essay kit 2; Biorad Laboratories B.V., Veenendaal, NL).

Indirect calorimetry

Prior to killing, resting metabolic rates (RMR) and daily energy expenditure (DEE) were measured for each animal in which antioxidant enzyme activity was measured, using an eight-channel indirect calorimetry system, as described by Oklejewicz *et al.* (1997). The mice were put in airtight chambers where oxygen consumption ($\dot{V}$ O_2, l h^{-1}) and carbon dioxide production ($\dot{V}$ CO_2, l h^{-1}) was measured simultaneously with ambient temperature and activity (passive infrared detectors). Oxygen and carbon dioxide concentrations of dried inlet and outlet air (drier: molecular siever 3 Å, Merck) from each chamber were measured with a paramagnetic oxygen analyzer (Servomex Xentra 4100) and carbon dioxide by an infrared gas analyzer (Servomex 1440). The system recorded the differentials in oxygen and carbon dioxide between dried reference air and dried air from the metabolic cages. The flow rate of inlet air was measured with a mass-flow controller (Type 5850 Brooks). Computerised data were collected every 10 minutes. All mice were measured for 24h at an ambient temperature of 22°C, mice from the cold group were measured for an additional 24h at 10°C. Oxygen consumption was calculated according the equation 2 of Hill

(Hill, 1972) to correct for volume changes with respiratory quotient below 1 and expressed in standard temperature and pressure. Metabolic rate (MR, kJ h^{-1}) was estimated using the following equation: MR= 16.18 x $\dot{V}$ O_2 + 5.02 x $\dot{V}$ CO_2 (Romijn and Lokhorst, 1961). Resting metabolic rate (RMR, kJ d^{-1}) was defined as the lowest value of metabolic rate calculated from cumulative means over 30 minutes. Daily energy expenditure (DEE, kJ d^{-1}) was calculated as the total metabolic rate over the 24-h measurement period.

Statistical analysis

Results are reported as means ± SEM. To test for effects of treatment and/or age ANOVA models in the MIXED procedure in SAS for Windows (version 9.1) were applied. Group, age, and group x age were added as fixed factors. Factors that may have influenced the outcomes were examined by including these as covariates in the models (i.e., food intake, body mass). Adjusted means were calculated by using the least squares means command in SAS MIXED. Data on antioxidant enzymes were $\log_{10}$-transformed as necessary to improve normality. Significance was set at $p \leq 0.05$.

RESULTS

Body mass & Food intake

Table 9.1 shows the characteristics of the mice housed under cold (10°C) and warm (22°C) conditions. Prior to the cold treatment there were no differences in body mass between groups (One way ANOVA: $F_{1,29}=1.71$, $p=0.279$). Mice housed under cold conditions had lower body mass at 3 and 27 months of age and in both groups body mass increased with age. Food intake differed between groups and with age; it was increased in COLD and aged animals.

Protein synthesis

Liver and muscle FSR decreased with age, i.e. between 3 and 27 months, but there was no main effect of COLD treatment (Table 9.1). In liver the age-related decrease was approximately 35% in COLD and 22% in WARM mice. These magnitudes differed as shown by a significant interaction effect between group and age. In muscle, the decreases in FSR with age averaged 26% and 39% in COLD and WARM mice, respectively. These responses were similar as shown by the lack of an interaction between group and age. Food intake can affect protein synthesis rates and was added to the models as a covariate to explore this relationship. In both liver and muscle food intake was not a significant covariate and adding food intake into the models as covariate did not substantially alter the effects of cold exposure or age.

Table 9.1. Effects of cold exposure on body mass, food intake, and fractional synthesis rates in liver and muscle.

	3 months		27 months			p-values		
Variable name	WARM	COLD	WARM	COLD	df	Group	Age	GxA
n	8	8	5	6				
Age (d)	98	98	833±1	836±1				
Body mass (g)	28.9±1.2	26.6±1.2	37.3±1.5	30.7±1.4	1,23	0.003	<0.001	0.122
Food intake (g d^{-1})	3.0±0.4	5.1±0.4	5.4±0.5	6.7±0.4	1,23	<0.001	<0.001	0.303
FSR liver (% d^{-1})	63.6±2.0	68.0±2.0	49.8±2.5	44.5±2.3	1,23	0.841	<0.001	0.039
FSR muscle (% d^{-1})	4.3±0.3	4.0±0.3	2.6±0.4	2.9±0.3	1,23	0.967	<0.001	0.320

Results for ANOVA are given in addition to least square (adjusted) means±SE for all groups. Bold values represent significant results ($p<0.05$). One mouse from the control group at 27 months was removed, because results indicated that the phenylalanine injection was not performed properly. n= sample size per group, df= degrees of freedom, GxA= GroupxAge interaction.

Antioxidant enzyme activity & Metabolism

Antioxidant enzyme activities in liver and heart of cold exposed and control mice are shown in Figures 9.1 and 9.2, respectively. The statistical analyses are shown in Table 9.2. Age affected SOD activity in both heart and liver. In both tissues, SOD activity increased between 3 and 11 months and decreased thereafter (Figure 9.1, top graph and Figure 9.2). The highest activity of hepatic GPx was observed at 3 months of age followed by a decline (Figure 9.1, bottom graph).

Cold exposure did not affect SOD activity in heart or GPx activity in liver, but did affect SOD activity in liver. This effect was mainly caused by a decrease in hepatic SOD activity in COLD mice compared to WARM mice at 19 months of age (Tukey test: $p<0.001$). In consequence, the COLD mice showed a faster decrease in SOD activity between 11 and 19 months of age (shown by a significant interaction between group and age, Table 9.2). For WARM animals the major decrease in activity occurred between 19 and 27 months.

Overall, resting metabolic rate (RMR, kJ d^{-1}) was (mean±sd) 44.2±.5.0 and 60.8± 8.8 and daily energy expenditure (DEE, kJ d^{-1}) was 57.1± 5.7 and 80.0± 5.7 in WARM and COLD mice respectively. RMR and DEE were highly increased at all ages in COLD mice (Two-way ANOVA, effect of group: $F_{1,50}=165$, $p<0.001$ for RMR and $F_{1,50}=372$, $p<0.001$ for DEE). RMR and DEE were added into the models as covariate to test whether they affected antioxidant enzyme activity. Neither RMR nor DEE predicted changes in enzyme activity within these models nor did they alter the effects of age or group. The first is also apparent in Figure 9.3 that shows the relationships between DEE and SOD activity in liver at all ages measured (none of which were significant; linear regression). Notice that variations in both metabolic rate and SOD activity were small in these inbred mice.

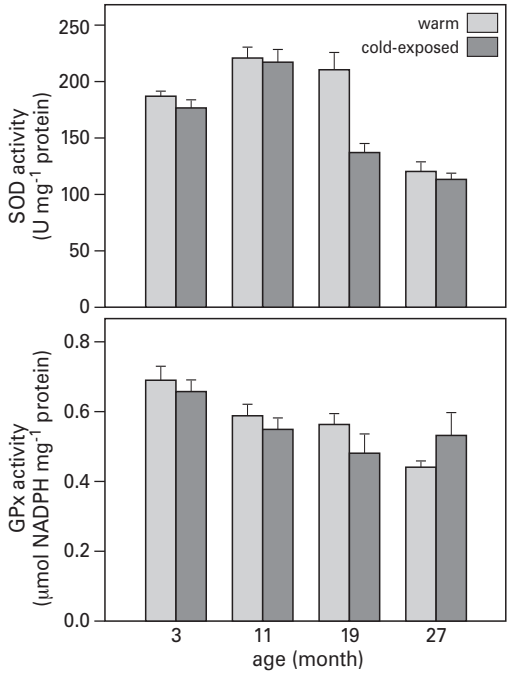

Figure 9.1. SOD (top graph) and GPx (bottom graph) activity in liver of mice exposed to cold (10°C) throughout their lives and of control mice (22°C) at different ages. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of pyrogallol auto-oxidation. One unit of GPx is defined as the amount of enzyme that oxidizes 1 μmol of NADPH per minute in the presence of reduced glutathione.

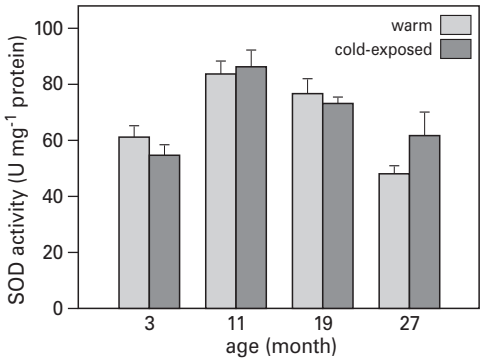

Figure 9.2. SOD enzyme activity in heart of cold-exposed (10°C) and control (22°C) mice at different ages. One unit of SOD was defined as the amount of enzyme that causes 50% inhibition of pyrogallol auto-oxidation. Values given are mean±sem.

Table 9.2. ANOVA on effects of group and age on antioxidant enzyme activities

		Group			Age			Group x Age	
Trait	N	d.f.	F	p	d.f.	F	p	F	p
SOD Liver	55	1,47	15.1	**<0.001**	3,47	50.7	**<0.001**	6.4	**0.001**
GPX Liver	53	1,45	0.2	0.47	3,45	18.9	**<0.001**	1.7	0.19
SOD Heart	57	1,49	0.5	0.68	3,49	7.2	**<0.001**	1.7	0.19

ANOVA models were performed in the MIXED procedure of SAS for Windows (version 9.1). Group, age and group x age were added as fixed factors. Data were $\log_{10}$-transformed as necessary to improve normality. N represents the total sample size and d.f. the degrees of freedom. Sample sizes were: at 3 months, n=8 and 7 respectively in warm and cold; 11 months, n=8; 19 months, n=7; 26 months, n=6. Liver samples of 2 mice were missing and for 2 other mice sample volume was too small to measure both SOD and GPx reducing the sample sizes for these measurements. Significant effects are in bold.

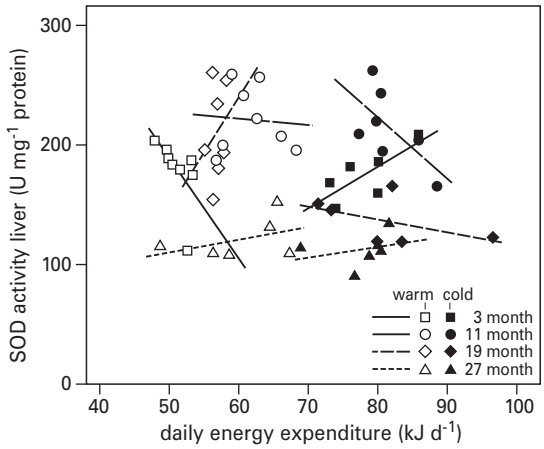

Figure 9.3. Relationship between daily energy expenditure and SOD activity in liver of warm (white symbols) and cold-exposed mice (black symbols) at various ages.

DISCUSSION

The primary purpose of this study was to examine the effects of cold exposure on age-related changes in protein synthesis and antioxidant enzymes in mice. In addition, the relationship between metabolic rate and antioxidant enzymes was explored.

In accordance with previous reports on rodents (mice: (Blazejowski and Webster, 1983; Vaanholt *et al.*, 2006), rats: (Lewis *et al.*, 1985)),decreases in fractional protein synthesis rates with age in all experimental groups and both tissues were found. On average, FSR decreased approximately 30% between 3 and 27 months in both liver and muscle. Studies in humans, however, have shown conflict-

ing effects of age on muscle protein synthesis, with either decreases (Short *et al.*, 2004; Yarasheski *et al.*, 1993; Young *et al.*, 1975) or no differences (Sheffield-Moore *et al.*, 2005; Volpi *et al.*, 2001). Such discrepancies between studies may have many causes, including muscle type used, sex, diet and activity level of subjects (Dorrens and Rennie, 2003). The technique used may affect the response, as the large (flood) dose procedure in fasted humans may stimulate protein synthesis (Cuthbertson *et al.*, 2005; Smith *et al.*, 1998). In fed animals, however, such responses are not observed (Rocha *et al.*, 1993) and this technique is routinely used in non-fasted rodent studies.

Long-term cold exposure (at 10°C) did not change protein synthesis rates in muscle or liver at 3 and 27 months of age. This is in agreement with several studies that have shown no change in muscle protein synthesis rates after long-term cold-exposure (rats (McAllister *et al.*, 2000; Samuels *et al.*, 1996), calves (Scott *et al.*, 1993), pigs (Lindsay *et al.*, 1988)), but contradicts observations in chickens where muscle protein synthesis rates increased (Yunianto *et al.*, 1997). The effects of cold exposure on protein synthesis are complex and probably depend on various factors, including the tissue measured, the intensity and the duration of the cold exposure, and the species studied. Furthermore, Scott et al (1993) have shown that (muscle) protein synthesis rates are influenced by food availability (Scott *et al.*, 1993). In cold-exposed calves that were food restricted, protein synthesis rates in muscle and skin were reduced but these were unaffected when calves were not food restricted. In the current study with mice fed ad libitum there was no change in muscle protein synthesis in response to cold exposure. Probably the 30% increase in food intake in the cold-exposed mice was sufficient to maintain levels of protein synthesis in muscle. Age (or period of development) may also be an important factor in determining the effect of cold exposure on fractional synthesis rates. Lewis *et al.* (1985) studied effects of caloric restriction on whole-body protein turnover in rats at different ages and found that the most pronounced response in FSR occurred at 12 months of age (increased by 45% compared to 6% at 24 months in calorically restricted mice vs. controls) (Lewis *et al.*, 1985). In the present study protein synthesis was only measured on 3 and 27 month old animals and at neither age was there an effect of cold exposure. Further studies would be necessary to determine if the mice were more sensitive at intermediate ages.

It has been hypothesised that increased rates of tissue protein turnover may contribute to heat production in cold-exposed animals (McAllister *et al.*, 2000) and this could have positive effects on survival. This is not supported by either previous reports (McAllister *et al.*, 2000; Scott *et al.*, 1993) or the current study. In contrast, protein synthesis rates in liver of the current cold-exposed animals decreased more with age than control mice kept at 22°C, and in muscle, the age-related change in protein synthesis rate did not differ between cold-exposed and control animals.

Age had a strong effect on antioxidant enzyme activity of SOD and GPx in murine heart and liver. An increase in SOD activity with a peak at 11 months of age and a subsequent decline was observed in both tissues. GPx activity in liver showed

a steady decline in enzyme activity from the age of 3 months onwards. Conflicting data exist on the effects of age on antioxidant enzyme activities (Gunduz *et al.*, 2004; Rao *et al.*, 1990; Sohal *et al.*, 1990; Tsay *et al.*, 2000; Vaanholt *et al.*, 2006), but comparison with other studies is complicated due to the use of different species, the choice of organs studied, and measurements at different ages (or only 2 ages). In studies where antioxidant enzyme activities were measured in mice heart and liver (see Chapter 7, Fig. 7.1 and 7.2) or rat brain (Tsay *et al.*, 2000) at multiple ages throughout life a similar pattern with age (with a peak at 10-12 months) was found as we show here for SOD activity. These data highlight the importance of measuring antioxidant enzyme activities at various ages when exploring developmental responses. We believe this will resolve many of the current discrepancies that appear to exist between different studies.

Long-term cold exposure increased metabolic rate by approximately 50% but did not affect antioxidant enzyme activity to early maturity (up to 11 months) in heart or liver. Data on SOD activity at 2 months of age are in agreement with those from voles of a similar age that had been bred and raised under cold conditions (~8°C) (Selman *et al.*, 2000). In that study, GPx and CAT activity were elevated in the heart of the cold-exposed voles. Other studies provide contradictory evidence on the effects of cold-exposure (Davidovic *et al.*, 1999; Kaushik and Kaur, 2003; Siems *et al.*, 1999; Spasic *et al.*, 1993) but again this may be a feature of the experimental conditions, including the tissue measured and the duration and/or intensity of the cold exposure. Age-dependent effects of cold-exposure on antioxidant enzyme activities have not been previously reported. Unexpectedly, the SOD activity in liver of cold-exposed mice (10°C) was, markedly decreased at 19 months of age compared with controls housed at 22°C. This may reflect either a higher susceptibility to oxidative stress or a lower production of free radicals at this age.

Despite a 50% increase in daily energy expenditure in cold-exposed animals throughout life, no major compensatory changes were observed in the antioxidant system in heart and liver tissue in these mice. Rather, SOD activity in the liver was even decreased at 18 months of age. CAT activity was not measured, and this may show compensatory changes occurred, as is observed in other studies (Kaushik and Kaur, 2003; Selman *et al.*, 2000). It has also been suggested that basal levels of SOD are sufficient to reduce the superoxide anion to hydrogen peroxide during moderate oxidative stress (Ji, 1999), and, if CAT was up-regulated, the available SOD and GPx might have been sufficient to cope with increases in radical production. Opposite to what we showed here (Figure 9.3), in exercising mice metabolic rate did significantly predict antioxidant enzyme activity (Vaanholt *et al.*, 2006). The low between-individual variation for both variables in this study may explain this discrepancy.

Increased protein turnover rates have been hypothesized to be an important factor contributing to the extension of life span in response to food restriction (Tavernarakis and Driscoll, 2002). The current study showed that protein synthesis and antioxidant enzyme activity decreased more steeply with age in the liver of

cold-exposed animals. This may cause cold-exposed animals to be more susceptible to ageing, because decreased levels of antioxidants would diminish the protection against ROS while lowered protein turnover will increase the half-life of proteins, enabling damaged proteins to accumulate in the cell and cause potential malfunction. However, cold exposure had no effect on median life span in rats (Holloszy and Smith, 1986). This would suggest that other compensatory changes (e.g. uncoupling) to reduce oxidative stress in response to high metabolic rates occur in cold-exposed animals. Uncoupling of mitochondrial respiration would be beneficial, particularly in cold-exposed animals, because energy is then dissipated as heat and this would markedly reduce oxidative stress (Brand, 2000; Erlanson-Albertsson, 2003; Speakman *et al.*, 2004).

In summary, long-term cold exposure did not result in compensatory changes in antioxidant enzyme activities in the heart and liver of mice or in protein synthesis rates in liver and muscle. Age strongly affected antioxidant enzyme activities and these showed either a peak at 11 months (SOD) or a gradual decline with age (GPx). Fractional protein synthesis rates also showed a decline with age in both liver and muscle. Numerous studies have shown that oxidative damage increases with age, and a decrease in antioxidant enzyme activity and/or protein turnover could explain this effect. Metabolic rate did not predict SOD and GPx levels in the animals in this study. Cold-exposed animals may have compensated for the high metabolic rates required to maintain constant body temperature by increasing the expression of uncoupling protein, thereby dissociating oxidative respiration from ATP production and reducing the generation of free radicals. Further study is required to determine whether this is the case.

Acknowledgements

We thank Suzan Anderson and David Bremner for performing the analysis of fractional synthesis rates and Annemieke Meijer for performing the antioxidant enzyme activity measurements. Serge Daan is thanked for commenting on earlier versions of the manuscript. L.M. Vaanholt was a recipient of an EU Marie Curie Training Site award to the Rowett Research Institute (Mass school), Aberdeen, Scotland.


Promovendus bijgespijkerd
LODEWIJK VANHOLT